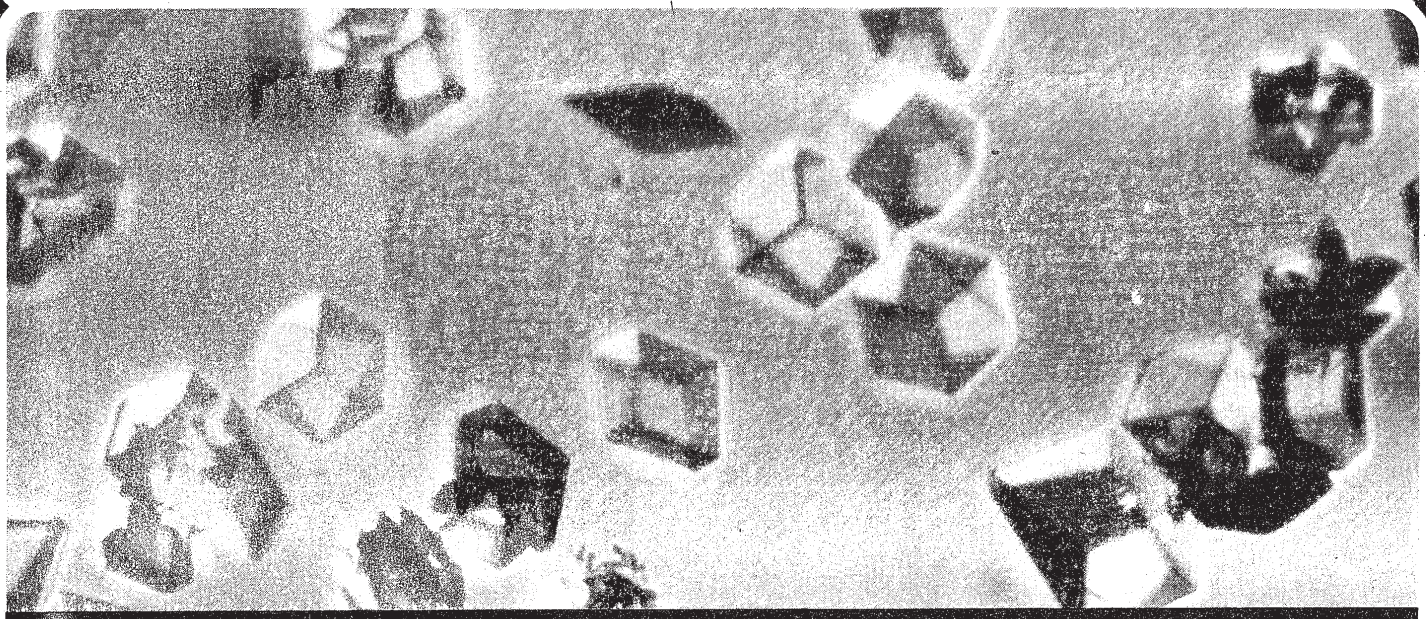
Sondernummer

MMW

Supplement 1/1983

S 1–S 166



Fortschritte in der Insulin-Therapie

Supplement-Herausgeber
F. Pfeiffer

IMPRESSUM

Anschrift:
MMW Medizin Verlag GmbH München, Neumarkter Str. 18, Postfach 80 12 46, D-8000 München 80, Tel. (0 89) 4 31 80-0, Telex 52 46 31 vervo.

Schriftleitung:
Prof. Dr. med. Werner Lang und Dr. med. Jochen Aumiller (presserechtlich verantwortlich). Anschrift wie Verlag.

Redaktion:
Dr. med. Jochen Aumiller (Chefredakteur), Dr. rer. nat. Till Uwe Keil (stellv. Chefredakteur), Hermann Lichtenstern, Dipl. sc. pol. Karsten Greller, Sabine Dehnert (Grafik). Anschrift wie Verlag.

Ständige Mitarbeiter der Redaktion:
Dr. med. O. Fenner, Hamburg; Prof. Dr. med. H. Holzgreve, München; Prof. Dr. med. G. Jörgensen, Göttingen; Claudia Meo, Paris; Dr. med. Ursula Sehrt, Mülheim.

Herausgeber des Supplementheftes:
Prof. Dr. Dr. h. c. mult. E. F. Pfeiffer, Zentrum für Innere Medizin der Universität, Steinhövelstr. 9, D-7900 Ulm.

Manuskripte:
Grundsätzlich werden nur solche Arbeiten angenommen, die vorher weder im Inland noch im Ausland veröffentlicht worden sind. Die Manuskripte dürfen auch nicht gleichzeitig anderen Blättern zum Abdruck angeboten werden.
Manuskripte von Autoren in nachgeordneter Stellung bedürfen der Zustimmung der zuständigen Leitung des Hauses.

Erscheinungsweise:
Die **MMW Münchener Medizinische Wochenschrift** erscheint wöchentlich in der MMW Medizin Verlag GmbH München.

Abonnement:
Bestellung beim Verlag, beim Buch- und Zeitschriftenhandel. **Bezugszeit:** Das Abonnement gilt zunächst für ein Jahr. Es verlängert sich um jeweils ein Jahr, wenn die Kündigung nicht bis 31. 10. im Verlag vorliegt. Aufnahme in Lesezirkel nur mit Zustimmung des Verlages. Bei höherer Gewalt und Streik besteht kein Anspruch auf Ersatz.
Bezugspreise: Einzelheft DM 7,50, Sammelausgabe (SA) DM 7,50 + Porto. **Jahresbezugspreis** DM 138,– (SA DM 148,–) – **für Studenten** DM 69,– (SA DM 74,–) zuzüglich jeweils **Versandkosten** Inland, Österreich u. Schweiz DM 42,–, Ausland DM 64,–. **Stabiler Ordner** für die SA mit 4-Loch-Spezialmechanik DM 28,–. **Index-medicus-Register** dazu DM 12,–. **Einbanddecken** (2 pro Jahrgang) jeweils DM 21,– zuzüglich Versandkosten.
Zusätzlich zu den Wochenausgaben können in unregelmäßiger Folge Supplementhefte erscheinen. Ihre Lieferung ist in den vorstehend genannten Jahresbezugspreisen nicht enthalten; sie werden auf Bestellung zum Einzelheftpreis zuzüglich Versandkosten geliefert.
Gerichtsstand und Erfüllungsort: München.

Anzeigenverwaltung:
Deutscher Ärzte-Verlag GmbH, Postfach 40 04 40, Dieselstraße 2, 5000 Köln 40 (Lövenich), Tel. 0 22 34/70 11–2 40, Telex 8 89 168. Z. Z. gilt Anzeigenpreisliste Nr. 21. Anzeigenschluß: 3 Wochen vor Erscheinen, für Kleinanzeigen 18 Tage vor Erscheinen. Anzeigenleiter: Eugen Volkert. Verantwortlich für den Anzeigenteil: Olaf Borchert, Anschrift wie Anzeigenverwaltung.

Herstellung:
L. N. Schaffrath, Grafischer Betrieb, Hartstr. 4–6, 4170 Geldern.

Two Routes for Producing Human Insulin Utilizing Recombinant DNA Technology

B. H. Frank, R. E. Chance

1. Human insulin synthesized in bacteria represents an important and safe source of highly purified insulin for the future treatment of the insulin-dependent diabetic.
2. Biosynthetic human insulin produced by recombinant DNA methods and prepared by either chain combination or conversion of proinsulin is chemically, physically, and biologically equivalent to pancreatic human insulin.

Zwei Wege zur gentechnologischen Gewinnung von Humaninsulin:
1. Die Synthese von Humaninsulin in Bakterien stellt eine wichtige und sichere Quelle zur Gewinnung von hochgereinigtem Insulin für die zukünftige Behandlung insulinabhängiger Diabetiker dar.
2. Biosynthetisches Humaninsulin ist chemisch, physikalisch und biologisch identisch mit pankreatischem Humaninsulin, gleich auf welchem Wege – entweder durch die Kombination getrennt hergestellter A- und B-Kette oder durch enzymatische Umwandlung von gentechnologisch hergestelltem Proinsulin – es gewonnen wird.

The preparation of human insulin utilizing recombinant DNA technology marks a significant accomplishment in the field of molecular biology and provides a secure source of insulin for the future treatment of the insulin-dependent diabetic. This manuscript will review some of the molecular biology that Eli Lilly has applied in order to accomplish this. Further, the procedures used to prepare and isolate highly purified human insulin are described. Finally, the results of analytical tests are presented to demonstrate that the human insulin produced using recombinant DNA technology is of high purity, is identical to the human insulin produced by the human pancreas, and is safe for use in humans.

The functioning and genetics of the bacterium, Escherichia coli, has been studied for many years, and, as a result, most of the recombinant this microorganism. For safety and containment reasons, Eli Lilly has chosen the K-12 strain of E. coli to use in our recombinant DNA research and production, because this is a weakened strain of E. coli which cannot colonize the intestinal tract of humans or animals. The functioning of the protein synthesis apparatus of bacterial cells is obviously central for being able to produce human insulin in these cells. Although proteins are synthesized only on the ribosomes in the cytoplasm of the cell, the genetic code for production of proteins resides in both the chromosomal DNA and in the small rings of cytoplasmic DNA called plasmids. Both of these sources of DNA are transcribed into messenger RNA, which is subsequently translated into proteins. The basis of recombinant DNA technology is our ability to manipulate this bacterial plasmid DNA which is acDNA, cleaving with restriction enzymes and inserting the desired DNA. The desired DNA is obtained either by synthesis, isolation from natural sources, or by a combination of these procedures. In the human insulin work, the A- und B-chain genes were prepared by synthetic nucleotide chemistry, while the human proinsulin gene was semisynthetic – that is, the gene was constructed using a segment of the natural DNA which codes for proinsulin along with a fragment of synthetic DNA. The nucleotide synthesis was performed by Itakura and coworkers at the City of Hope, and by Goeddel and coworkers at the Genentech Corporation (5, 9). After the desired DNA is obtained and inserted, the plasmid DNA is rejoined using a ligase enzyme and then reintroduced into the host cell thru a process called transformation. The cell then is cloned, that is many copies are made, and, after verifying that the desired and correct gene is present, the material is stored in ampoules for future use in production. Thus each fermentation is started from the same seed pool which has been verified to have the correct gene present.

In order to maximize the production of the desired protein in the E. coli cells, the gene message that was inserted into the plasmid also contains a so-called promoter. This promoter determines the rate at which messenger RNA is formed; thus, if one uses a strong promoter more messenger RNA is formed, and consequently there is greater production by the cell of the desired gene product. For human insulin biosynthesis, two promoter systems have been used, originally Beta-galactosidase and now tryptophan synthetase, or Trp E. The Trp E promoter yields more of the desired product as compared to the Beta-galactosidase promoter system. When the E. coli cells are producing

B. H. Frank, Ph. D., *R. E. Chance,* Ph. D., Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana 46285

Two Routes for Producing Human Insulin Utilizing Recombinant DNA Technology

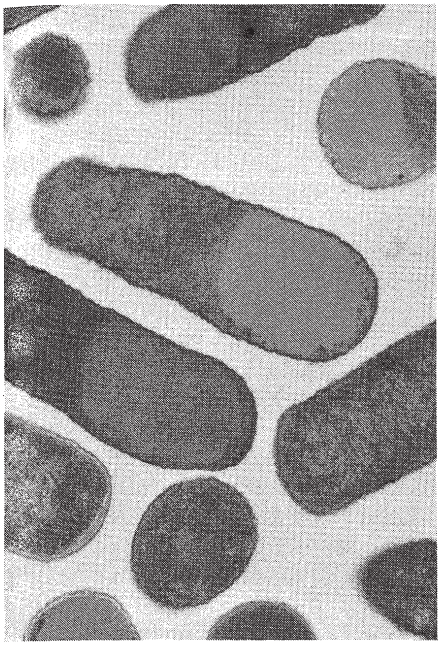


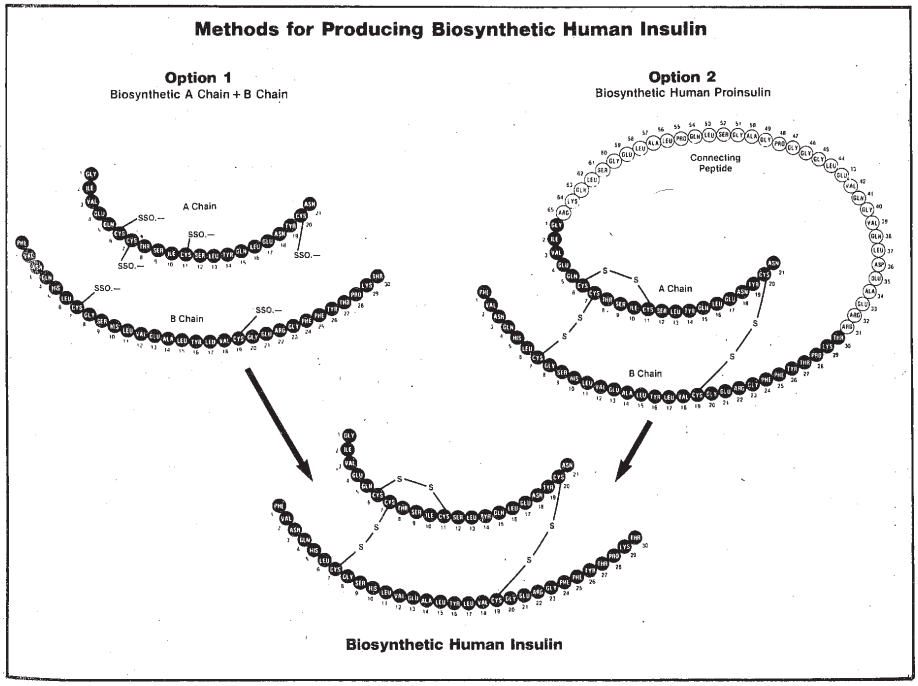
Figure 1: Transmission electron micrograph of inclusion bodies in E. coli cells of a culture producing Trp E-met-A-Chain chimeric protein. (Photograph kindly provided by Dr. D. C. Williams of the Lilly Research Laboratories).

the desired gene product, one can observe electron dense bodies (see Figure 1) which by immunocytochemical techniques (17) have been shown to be the promoter linked product (A chain or B chain or proinsulin) – called the chimeric protein. The chimeric product can be schematically represented as Trp E-Met-A Chain (or -B Chain or -Proinsulin). The methionine linkage provides a specific chemical cleavage site for release of the desired polypeptide from the promoter protein Trp-E.

An interesting fact to note is that all of the E. coli cells contain product, while in contrast, only a small fraction of the cells of the pancreas contain insulin. Thus, one actually has lesser amounts of impurities to remove during the isolation of the biosynthetic human insulin (BHI) as compared to the pancreatic insulins.

Figure 2 indicates the two schemes we have explored for producing biosynthetic human insulin. The current method is to make the A and B chains in separate E. coli fermentations, while the second route is the production of proinsulin in a single E. coli fermentation and eventually to transform it to human insulin. As far as we have been able to determine, both methods yield equivalent preparations of biosynthetic human insulin (2, 8).

Figure 3 illustrates in more detail the current method used to produce biosynthetic human insulin. The chimeric protein, Trp E-Met-Chain, is produced in the E. coli cells in separate fermentations. Methionine is used as a cleavage point since it is sensitive to the chemical cyanogen bromide, or CNBr, and because methionine does not occur either in the A or B-chains or proinsulin. After the cyanogen bromide cleavage, the A and B chains are converted to the stable S-sulfonate derivatives, purified and chemically combined to yield insulin. The insulin is then purified by modern gel-filtration and ion-exchange chromatographic procedures. At this point it should be emphasized that all of the biosynthetic human insulin presently being produced by Eli Lilly is derived from this chain combination procedure and that *all* clinical studies have been conducted with such insulin.



Before reviewing the characterization of the insulin produced by this process, a description of the chain combination procedure developed at the Eli Lilly Research Laboratories is of interest (2). This procedure consistently gives higher yields of insulin than ever reported for this reaction (see Figure 4). Optimal yields of human insulin are obtained using a 2:1 weight ratio of A chain to B chain in a 0.1 M glycine buffer at pH 10.5 and at 4°C. The S-sulfonate derivatives are reduced to sulfhydryl derivatives by use of nearly equivalent amounts of the thiol reducing agent, dithiothreitol, or DTT. The resulting solution is stirred for 24 hours at 4°C in an open vessel to permit the proper disulfide bonds to form as a result of air oxidation reactions. As shown, the insulin yield is approximately 60 percent relative to the limiting B chain. The biosynthetic human insulin is purified and isolated by column chromatography and crystallization. The excess chain materials and by-products are then recycled. This chain combination procedure is an extension of studies from several laboratories (7, 12, 13, 15) that were conducted during the 1960's when chemically synthesized chains of in-

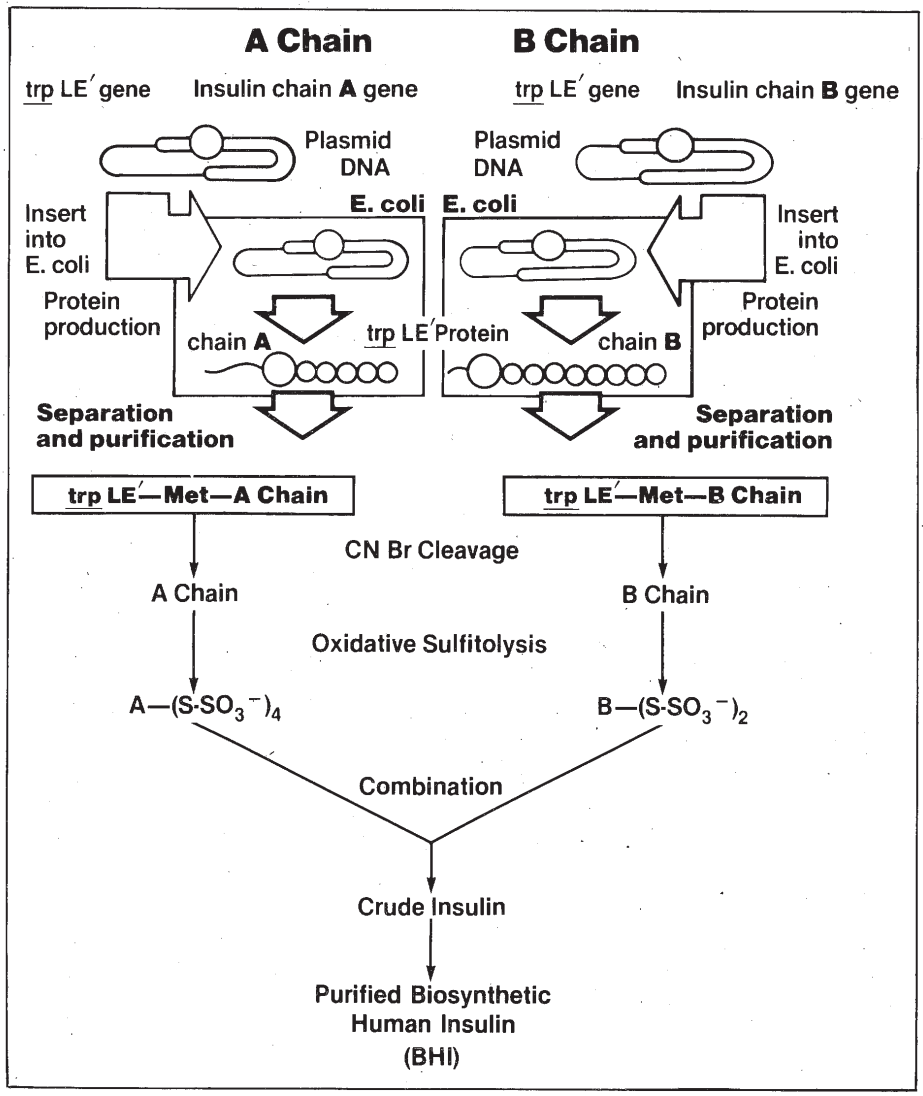


Figure 3: General biosynthetic and chemical modification process leading to the production of biosynthetic human insulin.

tic insulin preparations. However, the yields are significantly better than in the earlier studies due in part to the availability of modern analytical techniques such as high performance liquid chromatography (HPLC). The availability of this technique allowed the examination and optimization of the many variables of this complex series of reactions, and thus the achievement of excellent yields of human insulin.

The preparation of human insulin employing human proinsulin is shown in Figure 5. The chimeric protein produced by the E. coli cells is Trp E-Met-Proinsulin. As in the A tein is cleaved using cyanogen bromide. The proinsulin is subsequently converted to its S-sulfonate derivative by oxidative sulfitolysis and then isolated. Then the proinsulin-S-sulfonate is treated with a thiol reagent, beta-mercaptoethanol, which allows the proinsulin molecule to fold and form the proper disulfide bonds. Yields as high as 70 percent are achieved in this process, which was also developed in The Lilly Research Laboratories (8). The proinsulin is then purified by ion-exchange chromatographic methods and the by-products of the folding reaction are recycled. The proinsulin percent yield to insulin using a combination of the enzymes trypsin and carboxypeptidase B (see Figure 6). This process is a modification of the procedure originally developed by Kemmler and coworkers (14). The biosynthetic human insulin is subsequently purified by gel-filtration and ion-exchange chromatography, and by crystallization.

As indicated earlier, the biosynthetic human insulin produced via this scheme is identical to the insulin made by the chain combination method. Before turning to a discussion of the characterization of biosynthetic human insulin, we should point out that one of the exciting aspects of the proinsulin scheme is that we are now able to produce human proinsulin and C-peptide which may have interesting activities of their own and which can be investigated clinically for their potential usefulness in the treatment of diabetes.

The Characterization of Biosynthetic Human Insulin

A wide variety of evaluative tests have been used to exhaustively examine biosynthetic human insulin (see Table 1). The results of all of these tests can be best summarized by saying that biosynthetic human insulin has been shown to be chemically, biologically, immunologically, and physically identical to a native pancreatic human insulin standard (3). Tests in a variety of *in vivo* assays, as well as in many different insulin receptor assays, have demonstrated that biosynthetic human insulin exhibits equivalent biological activity to pancreatic human insulin. The same conclusion has been reached based on the data from the insulin radioimmunoassays as well as from the results of studies in the rabbit hypoglycemia test – it is obvious that biosynthetic human insulin is equivalent biologically to both pancreatic human insulin and porcine insulin.

The amino acid compositions of biosynthetic human insulin and por-

DOCKET ALARM

Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS
Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS
Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS
Sync your system to PACER to automate legal marketing.